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14. ABSTRACT

Wounds sustained by sharks and their skate and ray relatives have been observed to heal rapidly and without infection. A protective secretion produced by epidermal mucus cells in stingrays is being investigated to understand its role in the healing process and to identify mucus-associated antimicrobial compounds with the potential for development into novel therapeutics to treat wound infection pathogens. Freshly obtained mucus from two species of ray (cownose ray, Rhinoptera bonasus, and Atlantic stingray, Dasyatis sabina) contains at least 20 proteins/protein subunits based on gel electrophoresis under denaturing conditions. Chemical extraction of fresh mucus with 1) Tris-EDTA, 2) acetic acid followed by solid phase extraction, and 3) mild surfactants (Triton X-100, Tween 80, and N-octylglucoside) results in partial purification of mucus compounds. Fresh mucus also contains bacterial symbionts that are not seawater contaminants. Forty-six bacterial isolates cultured from cownose ray and 49 from Atlantic stingray epidermal mucus demonstrated antibiotic activity against at least one human pathogenic tester strain in primary screens performed at Mote Marine Laboratory. Of the 46 cownose ray isolates, 13 demonstrated antimicrobial activity against pathogenic bacterial tester strains when screened at University of South Florida Center for Biological Defense. Culturable libraries of all isolates have been cryopreserved.

15. SUBJECT TERMS
stingray epidermal mucus, antimicrobial activity, wound healing, wound infection pathogens, bacterial symbionts, culturable bacterial isolates

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INTRODUCTION:

Combat wounds, particularly blast wounds, are highly susceptible to infection and delayed healing. As a result, U.S. military caregivers are constantly seeking new antibiotics to treat soldiers wounded in combat arenas as well as those recovering in hospitals. Animal models of rapid wound healing can provide significant insights into novel approaches of treating combat wounds. There are numerous examples of remarkable wound healing in sharks and their skate and ray relatives in which traumatic wounds heal completely and quickly. Our hypothesis is that rapid and infection-free wound healing in elasmobranch fishes is related to antimicrobial activity present in epidermal mucus and that mucus-derived compounds will be effective against a variety of wound infection pathogens. The aims of this project are to identify antimicrobial activity against wound infection pathogens by epidermal mucus secretions of stingrays and skates, to determine the contribution of mucus to the rate of healing and resistance to infection in these fish, to establish biochemical profiles for mucus protein/peptide factors and to isolate antimicrobial compounds extracted from epidermal mucus. The studies to achieve these aims are coordinated among personnel at four institutions: Mote Marine Laboratory, Daemen College Center for Wound Healing Research, University of South Florida Center for Biological Defense, and Clemson University Animal & Veterinary Sciences. The anticipated research findings will impact the CMDRP Basic Research Program **Polytrauma and Blast Injury** project tasks directed toward **Wound Infection Prevention & Management** and **Antimicrobial Countermeasures** by identifying antibiotic compounds with the potential for development into novel antimicrobial agents that will facilitate the treatment of polymicrobial infections of combat-related wounds.

BODY:

Research during the initial year of this three-year funded project contributed information toward portions of most Tasks described in the SOW. Many of the Task objectives are planned to be continuous or on-going throughout the project. As a result, none of the Tasks has been completed. Since many of the Subtask activities build upon data acquired during the initial year's studies, some activities are further along while others are understandably in preliminary phases.

Research accomplishments associated with Tasks and Subtasks outlined in the approved Statement of Work are described below.

Task 1. Collect animals and epidermal mucus

Subtask 1a. Regulatory review and Animal Care and Use Review Office (ACURO) review and approval.
Protocols have received approval from both the USAMRMC ACURO and Mote Marine Laboratory IACUC.

Subtask 1b. Maintain MML IACUC-approved protocols
Because IACUC approvals at Mote Marine Laboratory are renewed annually, documentation of Mote's IACUC renewal was submitted to ACURO and received continued approval on 17 November, 2011.

Subtask 1c. Prepare animal containment facility (MML; Months 2- 6)
Preparation of animal containment facilities for the controlled experimental portions of the study is completed. Experimental tanks utilize temperature-controlled recirculating natural seawater maintained with biological and particle filtration and 12 hr on, 12 hr off photoperiods.

Subtask 1d. Collect animal specimens (MML; Months 4-30)
Collection of research animals will be an ongoing activity during the project. Cownose rays, *Rhinoptera bonasus*, and Atlantic stingrays, *Dasyatis sabina*, are collected passively, by surrounding them in shallow water with a seine net, transferring them with dip nets to an onboard live-well, and transporting them to Mote Marine Laboratory. Cownose rays are an active, schooling ray that require large holding tanks and are not ideal for long-term captive studies. Because of their relative size and local abundance, however, they can be sampled at time of capture and released unharmed, or can be maintained for a few days, sampled an additional time, and released. They have been useful for methods development during Quarters 1 and 2 of Year 1 and provide the opportunity for species comparisons of mucus composition. Atlantic stingrays are smaller than cownose rays, but because of their sedentary and solitary behavior, they are ideal for long-term captive maintenance in smaller tanks compared to those required for cownose rays, and can be easily manipulated for experimental procedures. This species of ray was used in experiments conducted during Quarters 3 and 4 of Year 1.

The third species planned for this project, clearnose skates, *Raja eglanteria*, will be included in Years 2 and 3.

Subtask 1e. Collect epidermal mucus from non-wounded and experimentally wounded animals (MML; Months 4 – 30).
Methods to collect epidermal mucus have been developed although improvements and optimization of procedures will continue to be incorporated. Epidermal mucus is sampled from individual rays by passive scraping of the pectoral fin surfaces with a sterile scoopula and transferred to sterile culture tubes (Figure 1).

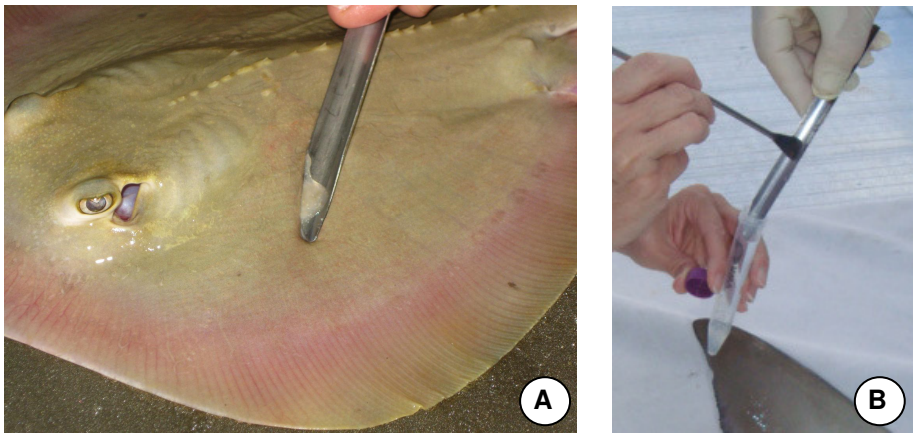


Figure 1. Epidermal mucus is collected by passive scraping of dorsal surface of an Atlantic stingray, *Dasyatis sabina*, **A**, and transferred to a sterile culture tube, **B**.

During Year 1, studies focused on characterizing mucus composition of two species of stingray (cownose rays, *Rhinoptera bonasus*, and Atlantic stingrays, *Dasyatis sabina*) under uncompromised (non-wounded) conditions. Mucus can be separated by gentle centrifugation into an aqueous supernatant and a viscous pellet (Figure 2), and can be processed as fresh samples, stored at 4 C for isolation of bacterial symbionts or extraction of proteins/peptides, or frozen at -80 C.

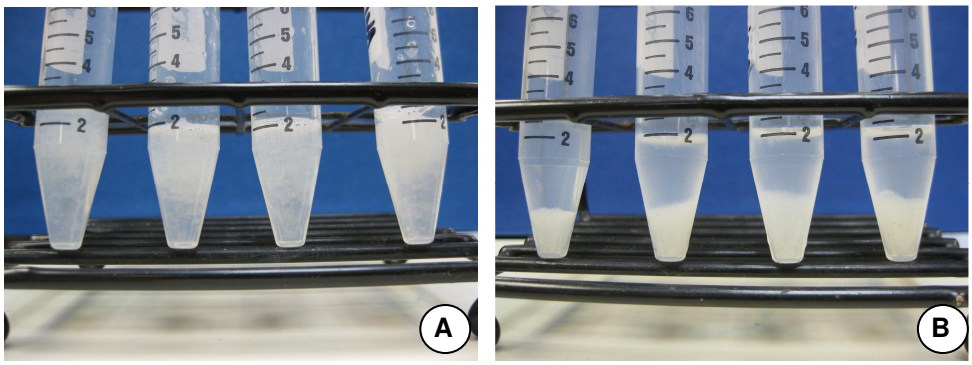


Figure 2. Fresh epidermal mucus before (**A**) and after (**B**) centrifugation, separating the sample into an aqueous supernatant and a mucus pellet.

Task 2. Determine antimicrobial activity of epidermal mucus

Subtask 2a. Prepare epidermal mucus extracts (MML; Months 4-30)
Freshly obtained cownose ray mucus samples were subjected to centrifugation (1,800 x *g* for 20 min at 4 deg C), resulting in an aqueous supernatant and a mucus pellet. Electrophoretic analysis using SDS-polyacrylamide gel electrophoresis (PAGE) indicates the aqueous supernatant of fresh mucus contains at least 20 proteins/protein subunits. Treatment of the mucus pellet with mild salt treatment (10 mM Tris:1 mM EDTA, pH 7.4), results in the extraction of very little additional protein (Figure 3).

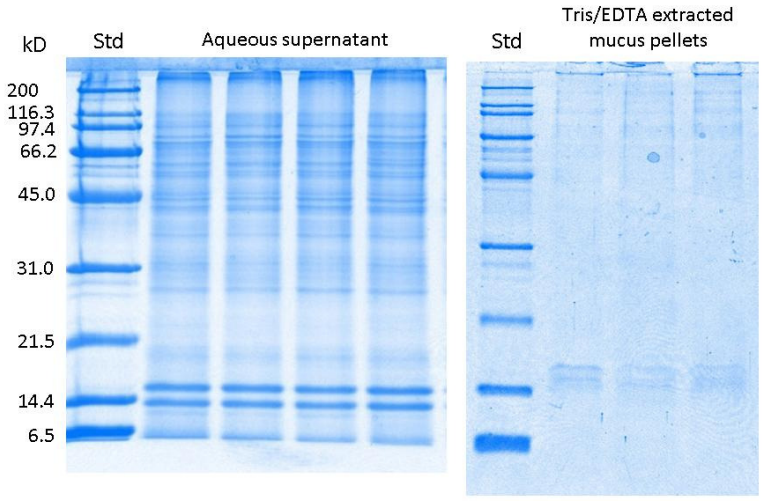


Figure 3. SDS 15% polyacrylamide gels of supernatant proteins/protein subunits from fresh mucus of four cownose rays, *Rhinoptera bonasus*, stained with Coomassie blue (left) and gels of proteins/protein subunits from Tris/EDTA extracted mucus pellets of three cownose rays (right) Molecular weights of standard proteins are in kilodaltons (kD).

As with cownose rays, aqueous supernatant fractions of freshly obtained Atlantic stingray mucus also contain numerous proteins. As visualized on SDS polyacrylamide gels (Figure 4), aqueous supernatant contains at least 20 proteins/protein subunits.

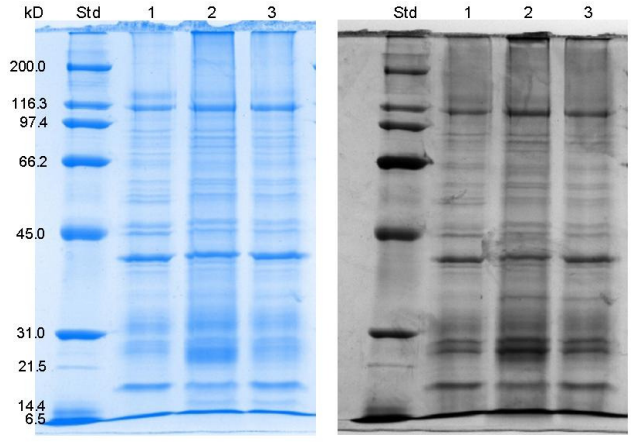


Figure 4. SDS 12% polyacrylamide gels of supernatant proteins/protein subunits from fresh mucus of three Atlantic stingrays, *Dasyatis sabina*, stained with Coomassie blue (left) and silver stain (right). Molecular weights of standard proteins are in kilodaltons (kD).

A flow diagram of proposed mucus extraction methods is summarized in Appendix 1. Initial procedures to perform 10% acetic acid extraction of the supernatant fraction and the mucus pellet of freshly obtained cownose ray mucus resulted in SDS-PAGE profiles of 15-20 proteins from the supernatant and as many as a dozen proteins from the acidic extracted pellet (Figure 5). Protein profiles among the aqueous supernatants, salt treated pellets, and acid extracted supernatants and mucus pellets were different in number of proteins and distribution of molecular weight bands.

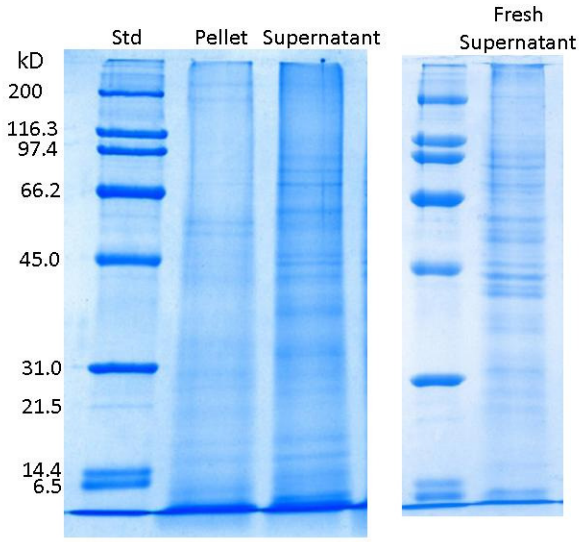


Figure 5. SDS 12% polyacrylamide gels of proteins/protein subunits from acid extracted mucus pellets and aqueous supernatant (left), compared to proteins in fresh supernatant (right).

Acetic acid extraction of aqueous supernatant and mucus pellets from Atlantic stingrays resulted in more protein extracted from the pellet fraction than from the aqueous supernatant (Figure 6), with most of the extracted protein being between 15 and 35 kD.

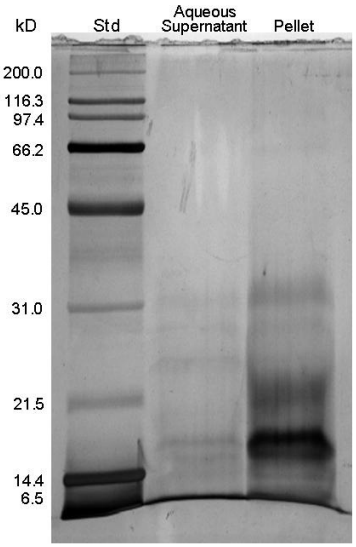


Figure 6. Silver stained SDS 12% gel of acetic acid extracted protein of aqueous supernatant and mucus pellets from freshly obtained Atlantic stingray mucus. Molecular weights of standard proteins are in kilodaltons (kD).

Portions of the aqueous supernatant fractions from Atlantic stingray mucus have been sent to co-PI's at Daemen College to begin looking at conditions to assess wound healing biomarkers, and to Clemson University to begin developing conditions for chromatographic separation of mucus compounds.

Extraction of Mucus Pellets with Nonionic Surfactants:
Methods were initiated to extract mucus pellets with three different chemical surfactants, Triton X-100, Tween 80, and N-octylglucoside. Triton X-100 is a nonionic surfactant with a hydrophilic polyethylene oxide group and a lipophilic hydrocarbon group. Tween 80 is a nonionic surfactant and emulsifier with hydrophilic polyether groups and an oleic acid-derived lipophilic group. N-octylglucoside is a surfactant frequently used to dissolve integral membrane proteins. Since such compounds are extremely useful in solubilization of molecules by dissociating aggregates and unfolding proteins, surfactant-treated mucus could result in fractions with different subsets of mucus proteins/peptides than those in aqueous supernatants or in acidic extracts.

Fresh mucus was collected from three cownose rays at time of capture. Samples were centrifuged and aqueous supernatants were aspirated. Resulting mucus pellets were washed with 3.0 mL of 0.2 micron filter-sterilized seawater, then recentrifuged. Each of the three washed mucus pellets was suspended in 2.0 mL of a 0.1% solution of one of the surfactants in Tris-buffered saline, pH 7.4. Suspensions were placed on a rocking platform and gently rocked for 2 h at room temperature, followed by centrifugation to obtain supernatants representing surfactant extracted proteins/peptides.

Surfactant extracts were subjected to stepwise fractionation using a series of PALL Nanosep centrifugal separators of decreasing molecular weight cutoffs (50 kD to 3 kD) resulting in a series of retentates and filtrates (Figure 1).

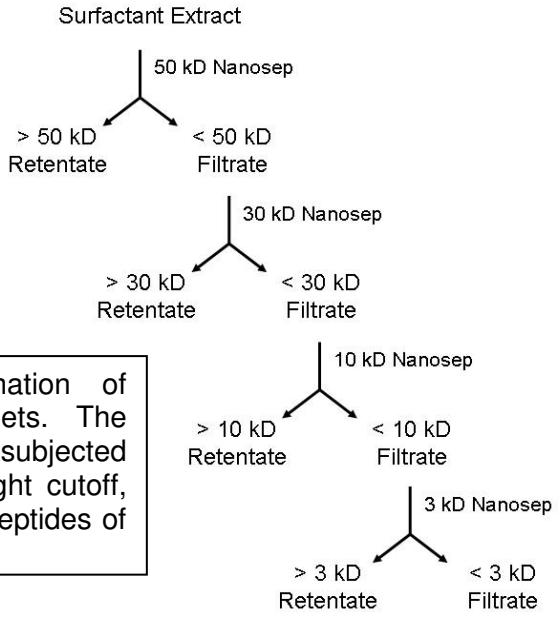


Figure 7. Stepwise fractionation of surfactant extracts of mucus pellets. The filtrate from each fractionation is subjected to the next lower molecular weight cutoff, until a filtrate containing protein/peptides of less than 3 kD is obtained.

Proteins in the resulting retentates and filtrates were separated using both non-denaturing and denaturing polyacrylamide gel electrophoresis (Figure 8).

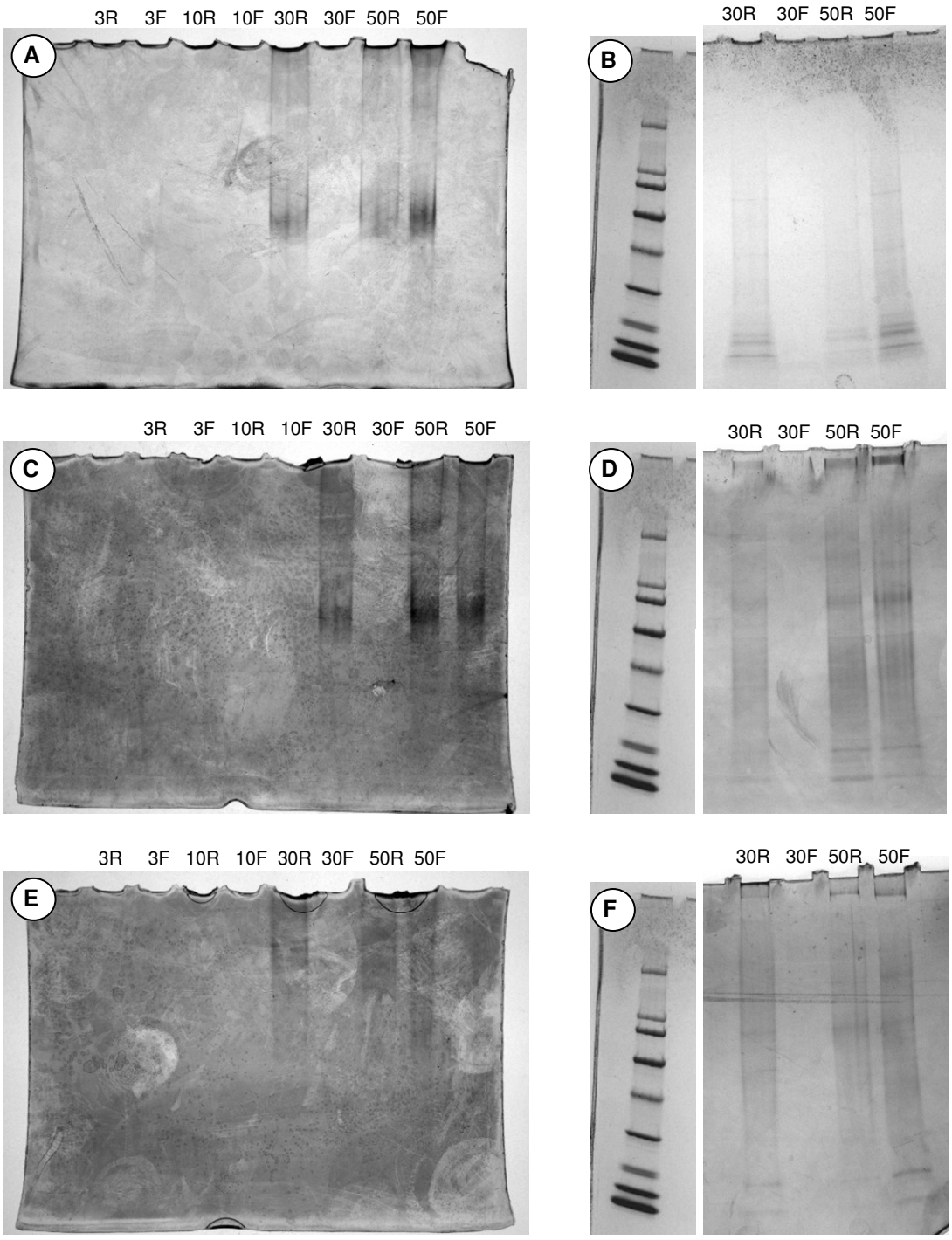


Figure 8. Silver-stained polyacrylamide gels showing surfactant-extracted mucus proteins under non-denaturing conditions (**A**, **C**, and **E**; 12% acrylamide, pH 9.0) and denaturing conditions (**B**, **D**, and **F**; SDS, Tris-glycine, 4-20% gradient gels). A and B, N-octylglucoside; C and D, Tween 80; E and F, Triton X-100. (R=Retentate; F=Filtrate).

Based on the non-denaturing gels, most of the native protein extracted by the surfactants was in the 30,000 to 50,000 molecular weight range (i.e., retained in the 50 kD retentate, 50 kD filtrate or 30 kD retentate). N-octylglucoside and Tween 80 (Figure 8, A and C) were more successful at extracting protein than Triton X-100. When subjected to denaturing conditions, gel bands representing protein subunits in the 5,000 to 15,000 molecular weight range were visible in the N-octylglucoside-extracted proteins (Figure 8B), while molecular weight bands from 5 to about 70 kD were visible in the Tween 80-extracted proteins (Figure 8D). Very little protein was extracted by Triton X-100 (Figure 8E and 8F).

Subtask 2b. Conduct primary antibiotic screens of epidermal mucus for antimicrobial activity (MML, USF)

Mucus extracts are not able to be assayed using the same agar overlay assay described for bacterial isolates (Task 3, Subtask 3b). Instead, a spectrophotometric assay is being developed to assay mucus extracts for antibiotic activity (Appendix 2). To screen mucus extracts prepared as described in Subtask 2a, one of the tester pathogens is grown overnight and a working stock of mid-log phase culture is prepared. Since most of the cownose ray mucus-associated bacterial isolates showed activity against *Bacillus subtilis* (Subtask 3b, Table 1), initial applications of the assay utilized this tester pathogen. With the absorbance at 600 nm of control cultures defined as no activity and a Penicillin/Streptomycin mix as an inhibitory control, the extent to which mucus extracts inhibit growth of the pathogen cultures is determined spectrophotometrically as a change in turbidity. Preliminary results from this assay are shown in Figure 9.

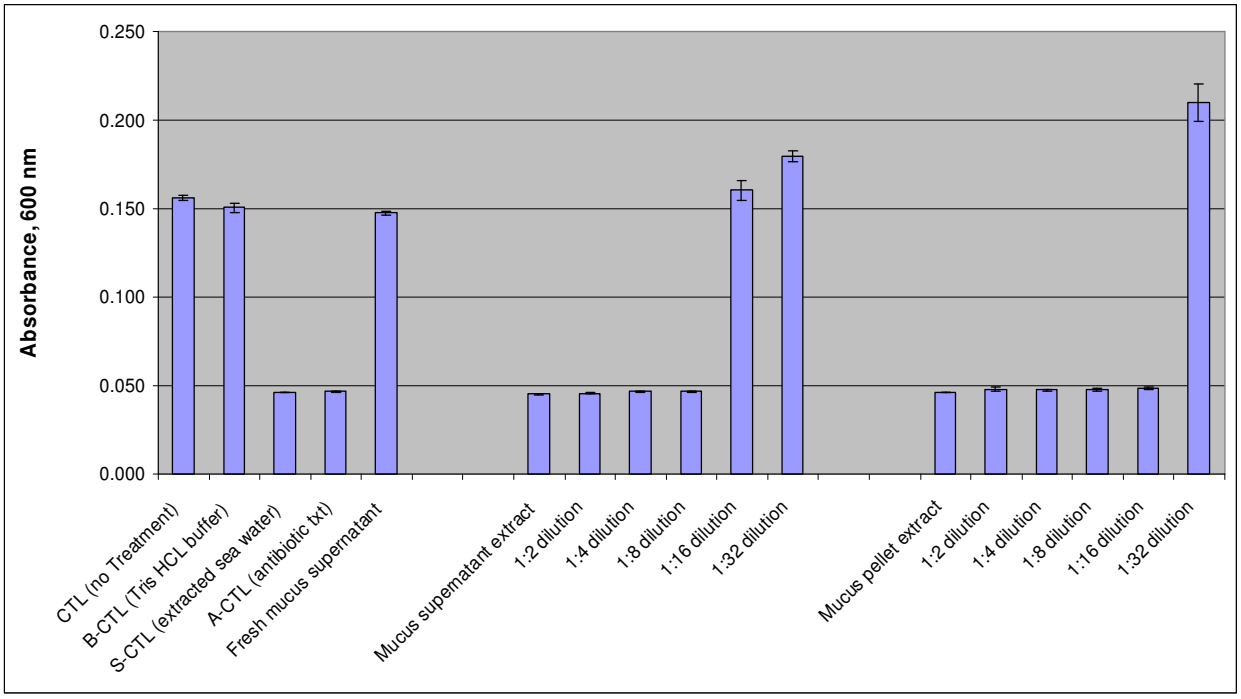


Figure 9. Preliminary results of spectrophotometric assay to evaluate mucus extracts for antibiotic activity. CTL, Control culture; B-CTL, Buffer control; S-CTL, Seawater control; A-CTL, Antibiotic control.

Although acidic extracts of both aqueous supernatants and mucus pellets showed activity against *B. subtilis* through multiple dilutions (through 1:18 dilutions for aqueous supernatant extracts and through 1:16 dilutions for mucus pellet extracts), it can not be ruled out that residual reagents from the extraction procedure could be responsible for the observed inhibitory activity, since inhibitory activity was also detected in extracted seawater controls, and no activity was observed with Tris-HCl buffer controls and fresh (unextracted) mucus supernatant.

Task 3. Separation of bacterial symbionts from epidermal mucus (MML, USF; Months 6-30).

Subtask 3a. Separate bacterial symbionts from epidermal mucus (MML; Months 6-24). Initial examination of cownose ray mucus revealed that fresh mucus samples contained approximately 2 x 10^8 colony forming units of bacteria/mL. Mucus pellets and aqueous supernatants from fresh samples were serially diluted in sterile seawater and plated onto marine agar (Sigma). Cultures were grown at ambient temperature for 3-5 days for development of bacterial colonies. Microorganisms exhibiting a unique colony or cellular morphology compared to other colonies on a single plate were subcultured to purification under the same set of growth conditions. Most of the mucus-associated microbial symbionts were isolated from the mucus pellet fraction. Purified isolates were cryopreserved at -80ºC in 96-well microtiter plate "culturable" libraries (Figure 10) and stored for antimicrobial screening.

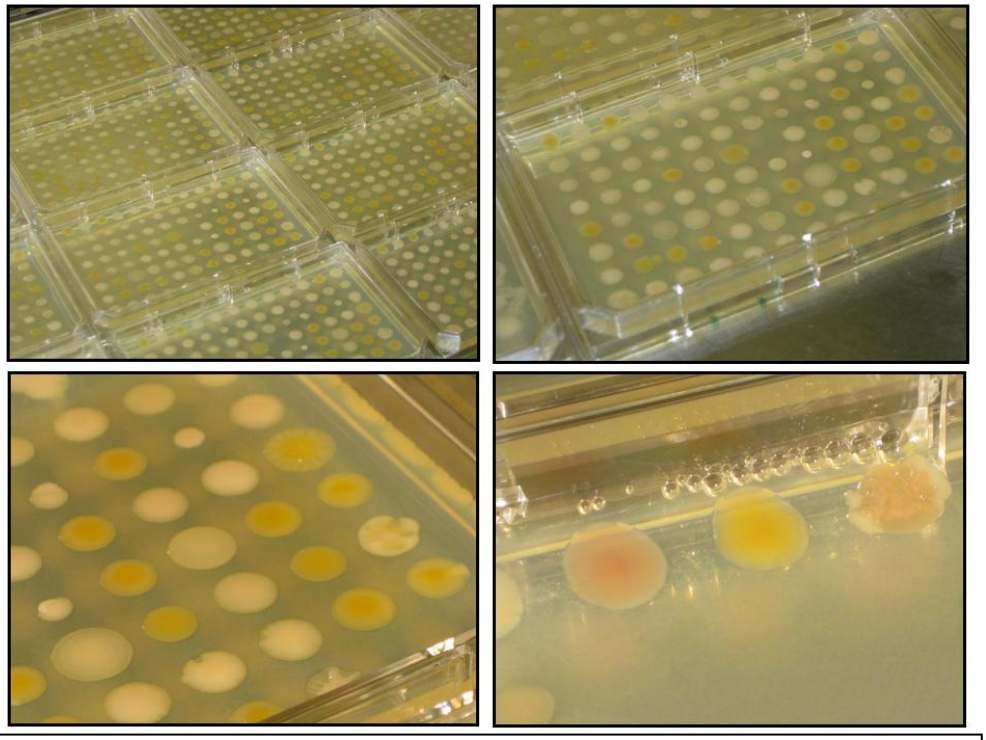


Figure 10. Representative subcultures of bacterial symbionts isolated from fresh epidermal mucus and plated in 96-well formats.

Subtask 3b. Screen bacterial symbionts for antimicrobial activity using primary screen tester strains (MML). **Part A**: cownose ray, *Rhinoptera bonasus*; **Part B**: Atlantic stingray, *Dasyatis sabina.*

Part A: Bacterial symbionts from cownose rays, *Rhinoptera bonasus*:
To date, 384 bacterial isolates have been cultured from cownose ray epidermal mucus. In primary screens performed at Mote Marine Laboratory (MML), 46 strains demonstrated antibiotic activity against at least one human pathogenic tester strain. All 46 strains with antibiotic activity have been purified, culturable libraries have been cryopreserved, and provided to project collaborators at the University of South Florida Center for Biological Defense, where they were screened against a different set of pathogenic bacterial strains (Subtask 3c).

For initial screens for antibiotic activity performed at MML, a simple method for the medium through-put identification of anti-infective molecules produced by marine bacterial symbionts has been developed (Appendix 3). This primary screen includes a panel of pathogenic bacterial tester strains including *Bacillus subtilis*, *Enterococcus faecalis*, Vancomycin resistant enterococcus (VRE), Methicillin-sensitive *Staphylococcus aureus* (MSSA), and Methicillin-resistant *Staphylococcus aureus* (MRSA).

Results of primary screens were achieved by growing each culturable isolate or environmental clone on the appropriate solid medium for two days at the appropriate growth conditions. Cultured libraries were then UV irradiated and overlayed with soft agar medium that had been inoculated with the target bacterial species. Growth inhibition of the target manifests as a zone of clearing around a colony that produces an active molecule. Bacterial isolates/environmental clones that inhibit growth of any of these pathogens are identified as potential producers of a lead compound**.** This comprehensive primary screen has been designed to help dereplicate and further classify anti-infective producers by setting aside potential repeats and by elucidating candidates that exhibit the broadest activity spectrum. Information gained during primary antibiotic screening will dictate potential targeting sources.

Subtask 3c. Screen extracts from bacterial symbionts for antimicrobial activity against wound infection pathogens (USF).

For antimicrobial screening at University of South Florida Center for Biological Defense (CBD), pathogenic bacterial tester strains included *Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus sp., Enterococcus faecalis, Listeria monocytogenes, Shigella boydii, Shigella sonnei, Shigella flexneri, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Acinetobacter baumanii,* and *Acinetobacter calcoaceticus.*

To screen for antibiotic activity, mucus-associated isolates from cownose rays (*R. bonasus*) were grown on marine agar or trypic soy agar for two days at room

temperature. Suspensions were made in either 1 mL marine broth or tryptic soy broth and adjusted to equal a 2 McFarland standard. 100 µL of each suspension was placed into a well of a microtiter plate. Using a 48 prong frogger, bacterial suspensions were inoculated onto marine agar media plates. Orientation of each plate was noted by use of wayson stain spot in one or two of the wells. Plates were left upright (media side down) overnight at room temperature. The next morning, the plates were turned over (agar side up) and left to incubate for another 24 hours. In the meantime, testing strains were grown on BA media and checked for purity and subbed so testing would use 24 hour growth. On testing day, the marine agar plates with the marine isolate spots were placed into the biosafety cabinet media side down and the lids taken off. The growth was exposed to UV light for 20 minutes and the plates were then closed and ready for use. One to five colonies of the testing strains were used to inoculate demineralized water and adjusted to match a 1 McFarland standard. Then 100 µL of that suspension was placed into 20 mL of melted (and cooled to 55-60°C) marine agar and slowly mixed by inversion two times. The agar/bacteria mixture was slowly poured over the marine agar plates containing the dead marine isolate spots and allowed to cover evenly and cooled. When the plates were cooled and the agar solidified, the plates were inverted and incubated at 35°C overnight.

Plates were examined for zones of inhibition of growth of the testing strains. The zone diameters were measured, and activity was described as any zone of no growth above and around the marine isolate colony spot. Inhibitory activity against *Acinetobacter baumanii* and *Staphylococcus aureus* demonstrated by two cownose ray mucus-associated bacterial isolates is shown in Figure 11.

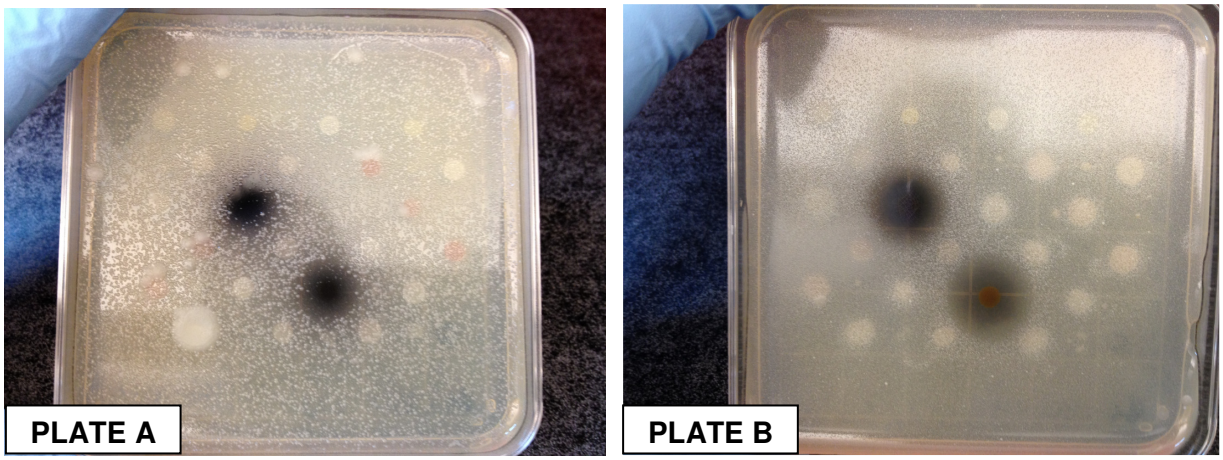


Figure 11. Zones of inhibition around spots #10 (MML Strain 505 F7, Table 1) and #19 (MML Strain 506 B3, Table 1) when tested with *Acinetobacter baumanii* (CBD 1323), **Plate A** and *Staphylococcus aureus* (CBD 44), **Plate B**.

A comparison of antibiotic activities for the 46 cownose ray mucus-associated bacterial isolates resulting from the MML and CBD screening procedures is shown in Table 1.

Table 1. Antibiotic activity of bacterial isolates from cownose ray (*R. bonasus*) mucus against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory (MML) and University of South Florida Center for Biological Defense (CBD).

Strain ID	Antibiotic Activity (MML)	Antibiotic Activity (CBD)
505 A6	*B. subtilis*	*B. cereus*
505 A7	*B. subtilis*	*B. cereus*, *L. monocytogenes*
505 A8	*B. subtilis*	
505 B4	*B. subtilis*	*B. cereus*, *L. monocytogenes*
505 C2	*B. subtilis*, VRE	
505 C4	*B. subtilis*	
505 D3	*B. subtilis*	
505 D7	*B. subtilis*, *E. faecalis*	
505 E7	*B. subtilis*	*B. cereus*
505 F7	*E. faecalis*, MSSA, VRE	*B. cereus*, MSSA, MRSA, *Micrococcus*, *E. faecalis*, *L. monocytogenes*, *Shigella*, *E. coli*, *S. enterica*, *Acinetobacter*
505 G1	*B. subtilis*	
505 G11	*B. subtilis*	
506 A2	*B. subtilis*	*B. cereus*
506 A3	*B. subtilis*	*E. coli*
506 A5	*B. subtilis*	
506 A8	*B. subtilis*	
506 A9	*B. subtilis*	
506 A12	*B. subtilis*	*B. cereus*
506 B3	VRE	*B. cereus*, MSSA, MRSA, *Micrococcus*, *E. faecalis*, *L. monocytogenes*, *Shigella*, *E. coli*, *S. enterica*, *Acinetobacter*
506 B5	*B. subtilis*	
506 B6	*B. subtilis*	
506 C5	*B. subtilis*	
507 A1	*B. subtilis*	
507 B10	VRE	
507 B12	*B. subtilis*	
508 A6	*B. subtilis*	
508 A8	*B. subtilis*	*B. cereus*, *E. faecalis*, *L. monocytogenes*
508 A9	*B. subtilis*	
508 B8	*B. subtilis*	*B. cereus*, *E. faecalis*, *L. monocytogenes*
508 C5	*E. faecalis*	
508 C8	*B. subtilis*	
508 C10	*B. subtilis*	
508 D1	MSSA	
508 D9	*B. subtilis*	
508 E5	*E. faecalis*, MSSA, VRE	
508 E9	*B. subtilis*	

508 E10	*B. subtilis*, VRE	
508 F4	*B. subtilis*	
508 F9	*B. subtilis*	
508 G6	MSSA	*Micrococcus*
508 G7	*B. subtilis*	
508 G8	*B. subtilis*	
508 G10	*B. subtilis*	
508 G11	*B. subtilis*	*B. cereus*, *S. epidermidis*
508 G12	*B. subtilis*	
508 H12	*B. subtilis*	

Subtask 3b. Screen bacterial symbionts for antimicrobial activity using primary screen tester strains (MML).

Part B: Bacterial symbionts from Atlantic stingrays, *Dasyatis sabina*:
To date, 227 bacterial isolates have been cultured from Atlantic stingray mucus pellets. In primary screens performed at Mote Marine Laboratory (MML), 49 strains demonstrated antibiotic activity against at least one human pathogenic tester strain. All 49 strains with antibiotic activity have been purified. Culturable libraries have been cryopreserved and provided to project collaborators at the University of South Florida Center for Biological Defense (Subtask 3c). Antibiotic activities for the 49 Atlantic stingray mucus-associated bacterial isolates resulting from the MML screening procedures are shown in Table 2. Data from USF related to activities of these isolates is not yet available.

Table 2. Antibiotic activity of bacterial isolates from Atlantic stingray (*D. sabina*) mucus against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory (MML). Diameters of zones of inhibition are included for comparison of relative sensitivities.

Strain ID	**Antibiotic Spectrum (Zone of Inhibition, mm)**
509B1	MSSA (2)
509D2	MRSA (1)
509D3	MRSA (0.5)
509F3	MRSA (1)
509F10	VRE (2)
509F11	EF (0.5), VRE (5)
509G1	EF (2), VRE (2.5), MSSA (2.5)
509G7	VRE (3)
509G8	VRE (4)
509H1	EF (1.5), VRE (2), MSSA (2.5)
509H12	EF (1.5), VRE (2), MSSA (2.5)

510A1	VRE (3.5)
510A4	VRE (1)
510A5	VRE (1.5)
510A6	VRE (2), EF (2)
510A7	VRE (1.5)
510B1	VRE (3)
510B4	VRE (3)
510B5	VRE (3), EF(1)
510B9	VRE (2.5)
510C1	EF (2), VRE (3)
510C3	VRE (1)
510C6	MSSA (2), VRE (5), EF (2)
510C9	MSSA (3), VRE (5.5), EF (1.5)
510C10	VRE (2)
510C11	MSSA (3), VRE (3.5), EF (2)
510D9	MSSA (2.5)
510D11	MSSA (1), VRE (4.5), EF (2.5)
510D12	MSSA (2), VRE (2), EF (1)
510E1	VRE (3), EF (3)
510E2	VRE (1.5), EF (1)
510E3	EF (2), VRE (2.5), MSSA (1.5)
510E4	VRE (1), MSSA (1.5)
510E8	VRE (2), EF (1)
510F9	VRE (2), EF (1)
510F10	VRE (2), EF (1)
510G1	VRE (3)
510G5	VRE (2.5)
510G9	EF (1)
510G11	VRE (2)
510H11	VRE (1.5)
511A8	MSSA (2), BS (3.5)
511A12	MSSA (3)
511B2	MSSA (2)
511B6	BS (2), EF (2), MSSA (2)
511B11	BS (1), EF (2), MSSA (2)
511C4	EF (2)
511C11	BS (1), MSSA (1.5)
511F9	MSSA (2), VRE (2.5), EF (1)

As evident from Table 2, 30 of the 96 bacterial strains assayed on Plate 510 demonstrated activity against vancomycin resistant enterococcus (VRE). (For assay details, please refer to Appendix 3.) Plate 510 is pictured in Figure 12.

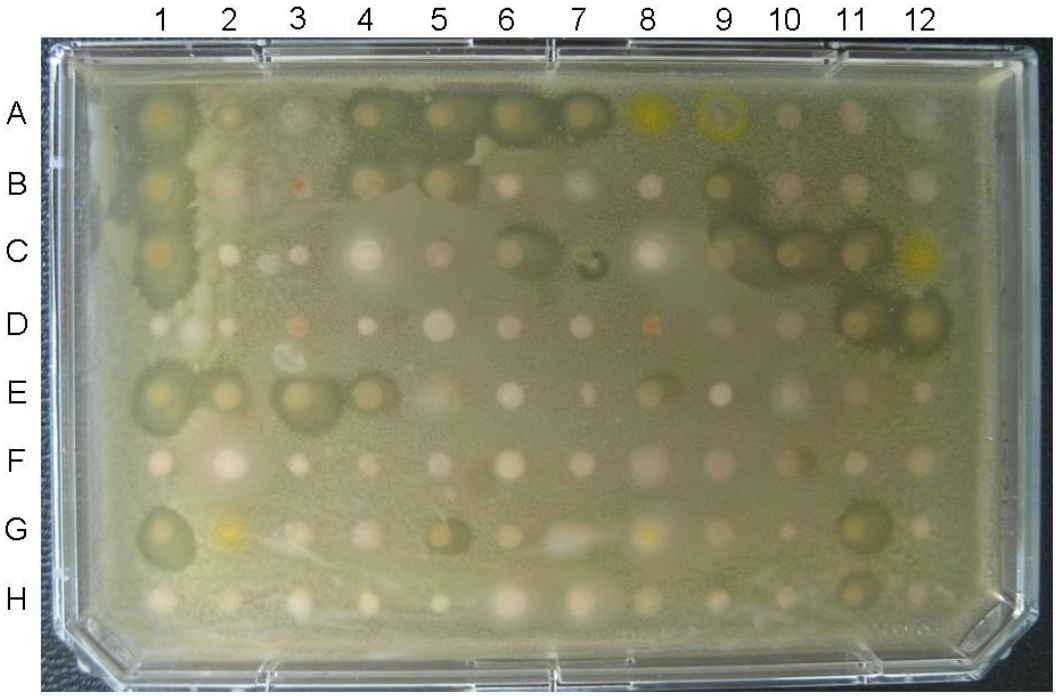


Figure 12. Photograph of Plate 510, containing 96 of the 227 bacterial isolates cultured from Atlantic stingray mucus overlaid with vancomycin resistant enterococcus (VRE). Zones of inhibition are visible in 30 of the 96 isolates assayed on this plate.

Bacterial identification. A portion of the pre-screened antibiotic producing bacteria isolated from Atlantic stingray mucus have been genetically identified. DNA was isolated from purified isolates using the MoBio Soil DNA isolation kit (MoBio, Inc). PCR amplification was carried out on genomic DNA with oligonucleotide forward primer U9F (5'-GAGTTTGATYMTGGCTC) and reverse primer U1502R (5'-GYTACCTTGTTACGACTT). Specific conditions for PCR amplification are detailed in Weidner et al. (1996). PCR products were electrophoresed on a 1% agarose gel, and verified using the AlphaImager 3300. An approximately 1,100 bp fragment was purified from the PCR reactions using the Qiagen PCR purification kit. PCR products were directly sequenced via BigDye TM terminator cycling and automated sequencing (University of Illinois) using R1n and U2 for forward and reverse strand synthesis (Weidner et al., 1996). Consensus sequences from forward and reverse strands were generated and GenBank BLAST searches were performed in order to demonstrate % identity to known bacteria available in the worldwide database (Altschul et al. 1997).

Six different genera were identified among 22 of the 49 *D. sabina*-derived isolates that produced antibacterial compounds against various tester strains (Table 3). Where only genus names are listed, the sequence was 100% identical to 2 or more different species within the genus. A different set of genetic or physiological criteria will be necessary for further identification of these isolates.

Some of the bacterial symbionts could be undescribed organisms, since 27 of the active isolates have not yet been genetically confirmed. Isolates that consistently exhibit a range of interesting antibiotic activities (as described by further screening at both MML and USF CBD) will be genetically verified and identities will be confirmed.

Table 3. Bacterial identities of antibacterial isolates from Atlantic stingray (*D. sabina*) mucus based on 16S rDNA sequence analysis.

Strain ID	Bacterial Identity
509 B1a	*Idiomarina* sp
509 B1b	*Pseudoalteromonas* sp
509 D3	*Vibrio* sp
509 F3	*Tenacibaculum* sp
509 F10	*Pseudoalteromonas* sp
509 F11	*Pseudoalteromonas* sp
509 G1	*Pseudoalteromonas ruthenica*
509 G7	*Pseudoalteromonas ruthenica*
509 G8	*Pseudoalteromonas ruthenica*
509 H1	*Pseudoalteromonas ruthenica*
509 H12	*Pseudoalteromonas ruthenica*
510 A1	*Pseudoalteromonas ruthenica*
510 A4	*Pseudoalteromonas ruthenica*
510 B1	*Pseudoalteromonas ruthenica*
510 B4	*Pseudoalteromonas ruthenica*
510 C1a	*Vibrio* sp
510 C1b	*Alteromonas* sp
510 C3	*Alcaligenes* sp.
510 E3	*Pseudoalteromonas ruthenica*
510 E4	*Pseudoalteromonas ruthenica*
510 H11a	*Pseudoalteromonas ruthenica*
510 H11b	*Idiomarina* sp
511 B6	*Pseudoalteromonas ruthenica*
511 B11	*Pseudoalteromonas ruthenica*
511 C11	*Pseudoalteromonas ruthenica*

Task 4. Determine contribution of epidermal mucus to wound healing in elasmobranchs (Months 6 - 30).

Subtask 4a. Experimental wounding of animals

Studies to characterize the wound healing process are in pilot study phases, with no preliminary data to report. In ongoing initial studies, experimental wounds are 1 square cm, penetrating the epidermal and dermal layers of the skin to the underlying musculature. At regular intervals, mucus in the area of the wound will be collected and digital photographs will be taken. Progression of healing of the wound area will be analyzed in comparison to a reference "target" using wound measurement software to calculate healing trajectories (Figure 13).

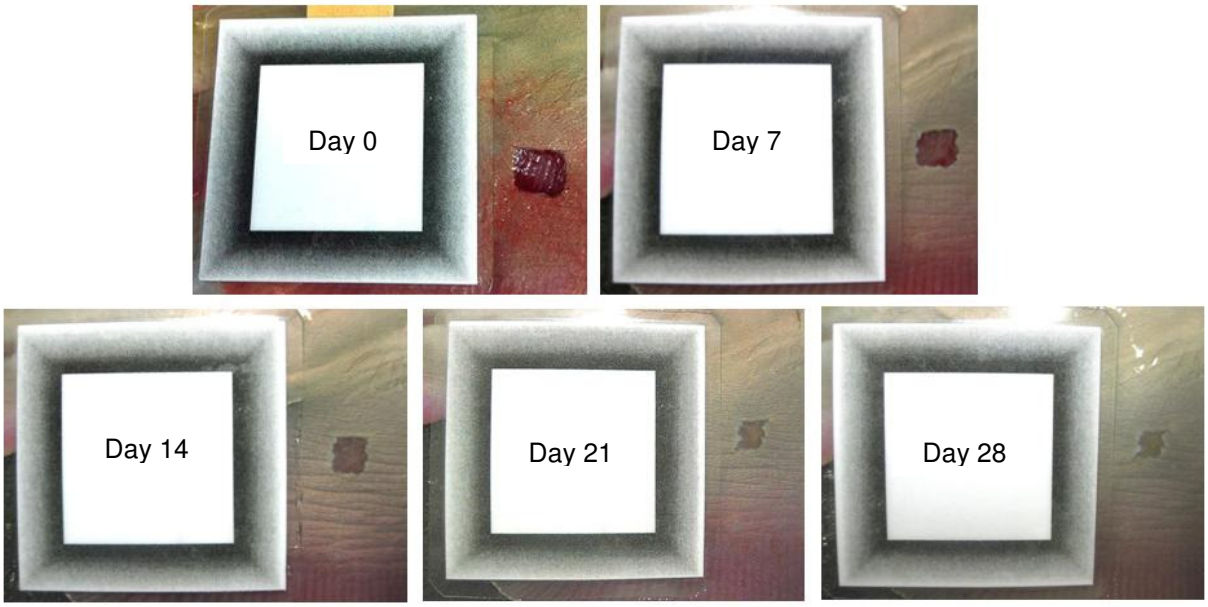


Figure 13. Experimental wound (approximately one square cm) through the epidermal and dermal layers of the skin of an Atlantic stingray, *Dasyatis sabina*, at weekly intervals for 4 weeks. Digital photographs are taken with an adjacent "target" to be used in the analysis of healing trajectories.

Task 6. Isolate bioactive compounds in epidermal mucus.

Subtask 6a. Isolate active components using chemical extraction and HPLC (high performance liquid chromatography) (Clemson)

Atlantic stingray mucus samples were provided to co-investigators at Clemson University to initiate methods development for the isolation and eventual identification of mucus compounds with antibiotic activity. Samples were centrifuged at 3,000 x *g*, then diluted 1:1 with 0.05 M ammonium bicarbonate and filter-sterilized through low protein binding 0.45 µ sterile filters.

The filtrate was fractionated using a series of molecular weight cutoff centrifugal ultrafilters (30 kD, 20 kD, and 10 kD). Retentates were resuspended in 0.05 M ammonium bicarbonate, pH 8, assayed for protein using absorption at 280 nm, and lyophilized. The lyophilizates were reconstituted to half the volume of original sample and analyzed on SDS polyacrylamide gel electrophoresis (Figure 14).

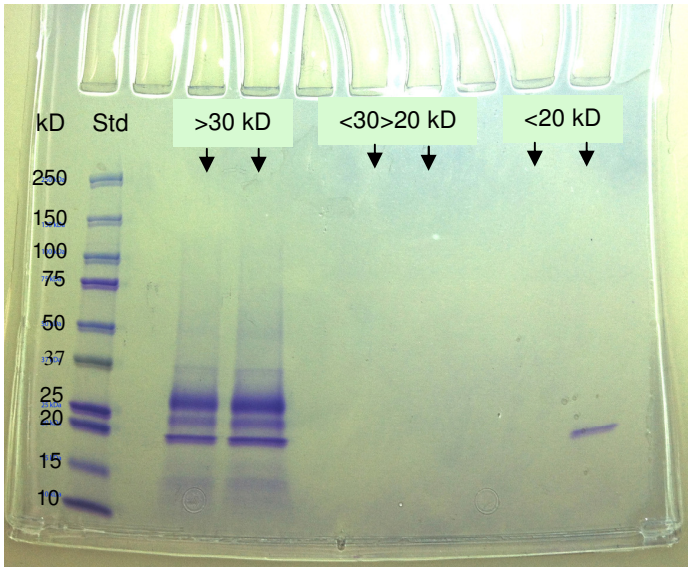
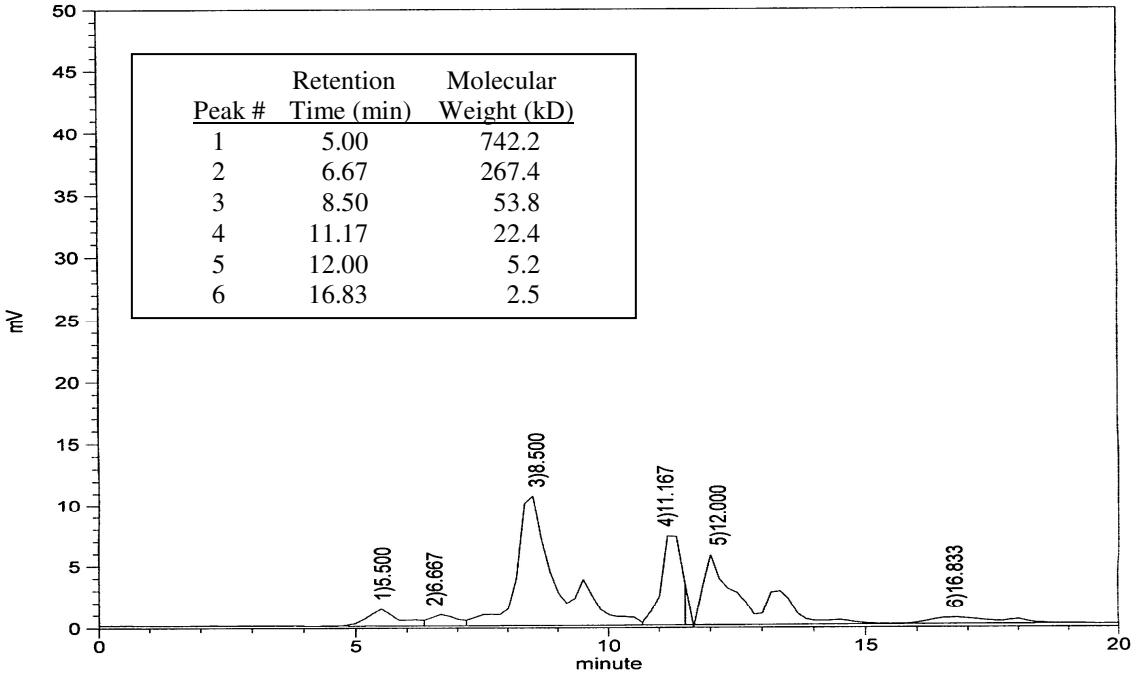


Figure 14. SDS 12% polyacrylamide gels of proteins/protein subunits showing protein/ protein subunit bands from three different molecular weight exclusion ultrafiltration retentates.

Three major subunit bands (~28-29 kD, ~23 kD, and ~17 kD), and a very faint band ~35 kD) resulted from analysis of a greater than 30 kD centrifugal ultrafiltration retentate. There were no visible bands in the less than 30 but greater than 20 kD ultrafiltrate, but a band at ~ 17-18 kD in the less than 20 kD ultrafiltrate.

The lyophilizate from the 10 kD retentate was subjected to separation using High Performance Liquid Chromatograpy (HPLC) on a Phenomenex BioSep gel filtration

column (Figure 15). Samples of the lyophilizate and fractions obtained from gel filtration were saved and will be assayed for antimicrobial activity using a modified Kirby-Bauer disk assay.



Peak #	Retention Time (min)	Molecular Weight (kD)
1	5.00	742.2
2	6.67	267.4
3	8.50	53.8
4	11.17	22.4
5	12.00	5.2
6	16.83	2.5

Figure 15. HPLC gel filtration analysis of *D. sabina* mucus samples. Phenomenex BioSep SEC 2000, 300 x 7.8 mm. Mobile phase:100 mM Phosphate Buffer, pH 6.8. Flow Rate: 1mL/min. Detection; 280 nm.

Preliminary experiments to isolate mucus components via chromatofocusing used a 7 to 4 pH gradient with polybuffer 74 HCl as the titrant. Elution of the greater than 10 kD retentate components equilibrated against 0.025 M imidazole, pH 7.4, indicated that the majority of the proteins were not retained on the 7.4 anion exchange and instead were voided. These experiments in the pH 7 to 4 range will be repeated with the partially purified gel filtration fractions. In addition, chromatofocusing in a 9 to 6 pH gradient using polybuffer 96 HCl will be performed.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed methods for passive sampling of epidermal mucus from two species of rays, *Rhinoptera bonasus* (cownose ray) and *Dasyatis sabina* (Atlantic stingray).

- Characterized epidermal mucus as being composed of an aqueous supernatant containing at least 20 proteins or protein subunits and a viscous pellet containing significant amounts of symbiotic bacteria that are NOT seawater contaminants.

- Methods being developed to extract mucus compounds include mild salt (Tris-EDTA), acid (acetic acid/solid phase extraction) and mild surfactants (Triton X-100, Tween 80, and N-octylglucoside).

- 384 bacterial isolates purified from *R. bonasus* epidermal mucus. In primary screens performed at Mote Marine Laboratory, 46 of the 384 strains showed antibiotic activity against at least one human pathogenic tester strain. Of these, 13 strains demonstrated antibiotic activity against at least one wound infection pathogen strain in screens performed at University of South Florida Center for Biological Defense. Two strains were active against *Acinetobacter*.

- 227 bacterial isolates purified from *D. sabina* mucus. In primary screens performed at Mote Marine Laboratory, 49 of the 227 strains demonstrated antibiotic activity against at least one human pathogenic tester strain. 36 strains were active against VRE, while 24 strains were active against two or more tester pathogens.

- GenBank BLAST searches identified six different genera among 22 of the 49 *D. sabina* bacterial isolates that produced antibacterial compounds against various tester strains. Unidentified strains may represent undescribed bacteria.

REPORTABLE OUTCOMES:

Presentations

- MIDRP/DHP Wound Symposium meeting 2-4 May 2010 in San Antonio, TX. "Novel Compounds from Stingray Epidermal Mucus with Antimicrobial Activity Against Wound Infection Pathogens"

- 10th Meeting of the Consortium of Biodefense Researchers, September 12-13, 2011, University of South Florida, Tampa, FL "Evaluation of Antimicrobial Activity of Topical Secretions from Stingrays"

Request for Research Data

- Provided Powerpoint slides to Jessica D. Eisner, MD, Deputy Director, Military Infectious Disease Research Program for portfolio presentation of the bi-annual research update for the staff of the Office of the Asst. Secretary of Defense, Health Affairs [OASD(HA)], scheduled for 14 October, 2011

Databases

- Using the worldwide database GenBank, BLAST searches were performed on 16S rDNA sequence data generated from epidermal mucus
- Six different genera of bacteria that produce antibacterial compounds against various tester strains have been identified from among *D. sabina* mucus-associated bacterial isolates
- Many 16S rDNA sequences did not match sequences in the existing database

CONCLUSIONS:

Epidermal mucus collected passively from the surface of stingrays can be separated by gentle centrifugation into an aqueous supernatant and a viscous pellet. As visualized on SDS polyacrylamide gels, aqueous supernatant contains at least 20 proteins/protein subunits, acidic extracts of pellets and supernatants contain 15-20 proteins/protein subunits, while surfactant extracts contain 6-8 proteins/protein subunits. Molecular weights range from 6,000 to 200,000 daltons. Methods to assess antimicrobial activity of chemically extracted material have been hampered by difficulty in removing residual chemicals (i.e., acids and organic solvents) involved in extraction procedures. Future work to address this problem will focus on additional steps clean-up steps, such as dialysis, lyophilization, and evaporation.

Epidermal mucus also contains a natural flora of symbiotic bacteria, which tend to be concentrated in the mucus pellet. Culturable bacteria from the mucus of two species of stingray (cownose rays, *Rhinoptera bonasus*, and Atlantic stingrays, *Dasyatis sabina*) have been isolated and screened for antimicrobial activity at Mote Marine Laboratory (MML) using a primary screening panel of pathogenic bacterial tester strains, including *Bacillus subtilis,* Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA), *Enterococcus faecalis*, and Vancomycin-resistant *Enterococcus* (VRE). Of 384 bacterial isolates cultured from cownose ray epidermal mucus, 46 strains demonstrated antibiotic activity against at least one human pathogenic tester strain. Of 227 bacterial isolates cultured from Atlantic stingray mucus, 49 strains demonstrated antibiotic activity against at least one human pathogenic tester strain.

Mucus-associated bacterial isolates have also been provided to project co-investigators at University of South Florida Center for Biological Defense (CBD), where cultures have been screened against a different panel of human pathogenic tester strains, including *Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus sp., Enterococcus faecalis, Listeria monocytogenes, Shigella boydii, Shigella sonnei, Shigella flexneri, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Acinetobacter baumanii,* and *Acinetobacter calcoaceticus.* Of the 46 cownose ray mucus-associated bacterial isolates demonstrating antibiotic activity from the MML screening procedures, 13 strains demonstrated antibiotic activity against at least one human pathogenic tester strains in the CBD panel.

Evaluation of the knowledge:

Since this funded program focuses on basic research, development of a medical "product" is not within the scope of this project. However, the antimicrobial activity demonstrated by numerous mucus-associated bacterial isolates holds great promise for the identification of antibiotic compounds and future development of therapeutics to treat wounds sustained on the battlefield.

REFERENCES (format used in *Military Medicine*)**:**

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25:3389-3402.

Weidner S, Arnold W, Pühler A: Diversity of uncultured microorganisms associated with the seagrass *Haliphilia stipulacea* estimated by Restriction Fragment Length Polymorphism analysis of PCR-amplified 16S rRNA genes. Appl Environ Microbiol 1996; 62: 766-771.

APPENDICES:

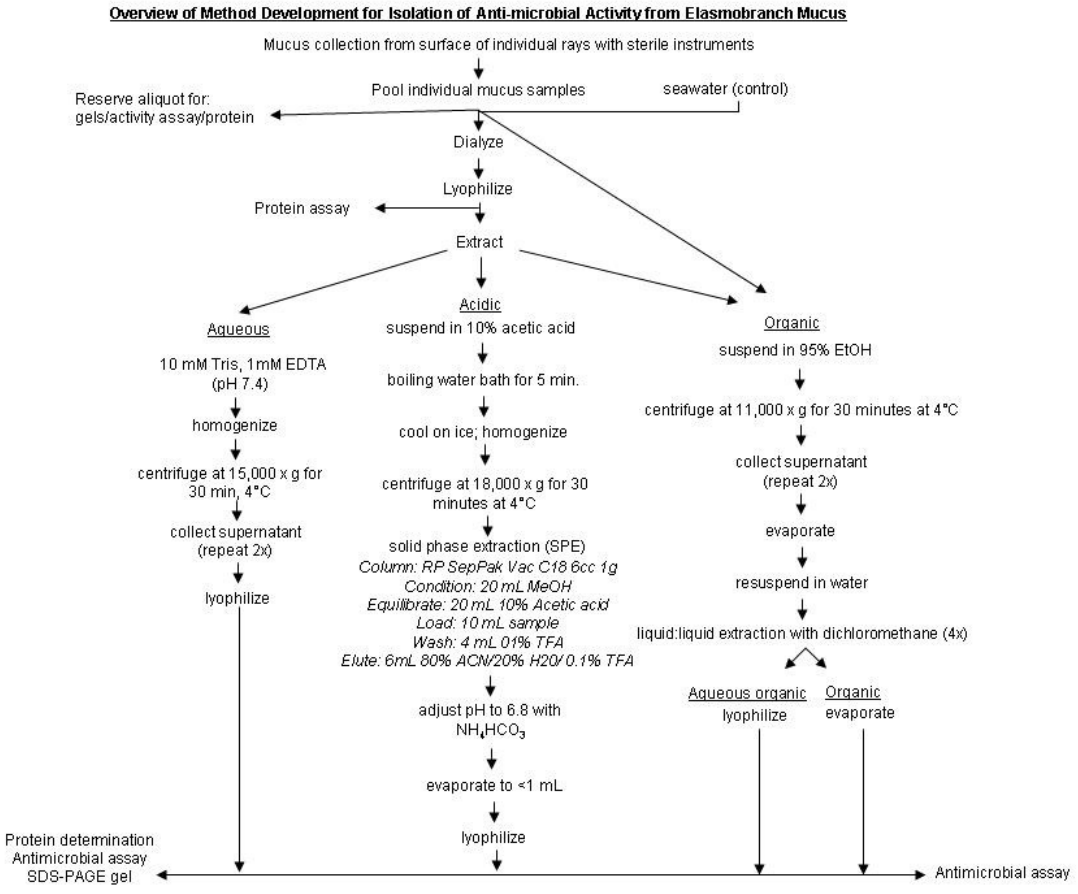
Appendix 1. Flow diagram of proposed mucus extraction methods

Appendix 2. Spectrophotometric antimicrobial assay: *Bacillus subtilis*

Appendix 3. Antibiotic screening of cultured libraries: Procedure

Appendix 4. Meeting abstract

Appendix 1. Flow diagram of proposed mucus extraction methods



Appendix 2. Spectrophotometric antimicrobial assay: *Bacillus subtilis*

1) Grow overnight of *Bacillus subtilis* at room temperature, shaking (one to three colonies of actively growing plate cultures into 5 mls of Luria Broth).

2) To prepare a working *B. subtilis* stock, pellet 3 mls of mid-log phase cultures (A_{600} of 0.4 ± 0.1) by centrifugation at 500*g* for 10 minutes at 4ºC. Wash 2 times in sterile PBS. Resuspend final washed pellet to A_{600} of 0.1

3) Add 10 µl of *B. subtilis* stock to 1 µl of diluent control or diluted antibacterial protein sample in duplicate microcentrifuge tubes. Mix by pipette and incubate at 25ºC for 30 min.

   NOTE: 6.2 µg/ml of Penicillin/Streptomycin mix is adequate to inhibit growth of *B. subtilis* for a control.

4) Add 489 sterile TSB to each tube, mix, and aliquot 100 µl into 96-well micro titer plate in quadruplicate. Incubate plate at 25ºC (potentially incubate a duplicate plate at 37ºC for comparison, assay for loss of activity at higher temp) until control wells reach an $A_{600}$of 0.08 to 0.120. RECORD DATA.

Appendix 3. Procedure for antibiotic screening of cultured libraries (Mote Marine Laboratory):

Bacteria are plated from libraries onto rectangular single well plates containing GASWA, Marine Agar, or other appropriate media. At least 2-3 days growth is required at room temperature to grow organisms to sufficient sized "colonies" for assays. The night before antibiotic assays are to be performed, cultures of tester strains are started. Overnight growth is done at 37ºC with gentle agitation.

On the day of (or the day before) the assays, rectangular plates with cultured libraries are UV-irradiated to kill the colonies being tested. This eliminates cross-contamination when overlaying with the tester strains.

0.8% agar overlays (LB, TSB, GASW and marine broth overlays) for tester strains are prepared, autoclaved, and placed in an incubator at 42ºC with gentle agitation.

Place plates (without lid) in the hood and turn on the UV lamp. Irradiate on high for 15-30 minutes (UV resistance may vary depending on the source of bacteria used in library generation). Mark the plates that are irradiated in some way with a marker to indicate they have been "killed."

1. Assay Set Up:
    a) Remove appropriate agar from incubator
    b) Inoculate with appropriate amount of batch culture (amounts may vary depending on growth stage).

2. Using glass pipette transfer 10ml of 0.8% agar from container to one well plate, move back and forth along center or empty edge of plate dispensing agar, then tilt the plate to distribute, ensure complete covering. Have appropriate tubes, UV irradiated plates, and pipettes warm in the 42ºC incubator.

3. Incubate library plates overnight at 30º C.

4. Identify zones of inhibition and note width from edge of colony using calipers. Record data for each strain in Excel file using frozen storage library grid. Data entered include tester strain active against and diameter in mm of zone of inhibition for each tester strain noted.

Appendix 4. Meeting abstract

Military Infectious Diseases Research Program (MIDRP)
Defense Health Program enhancement (DHPe)
Wound Symposium 2011, 2-4 May 2011
Holiday Inn – Riverwalk, San Antonio, TX

Novel Compounds from Stingray Epidermal Mucus with
Antimicrobial Activity Against Wound Infection Pathogens
DM102080
Carl A. Luer, Ph.D.
2011

There are numerous examples of remarkable wound healing in sharks and their skate and ray relatives in which traumatic wounds heal completely and quickly. These fishes naturally secrete protective epidermal mucus that increases in response to injury or other stress. With goals of determining the contribution of mucus to wound healing and resistance to infection, initial studies have focused on characterizing mucus composition under natural, uncompromised conditions. Preliminary analysis indicates the aqueous supernatant of fresh mucus contains at least 20 proteins, while acidic extracts contain approximately 15-20 proteins. Initial assessment of microbial symbionts indicates that fresh mucus contains approx 2 x 10^8 colony forming units of bacteria/mL. These data will contribute to baseline information for studies in which animals will be experimentally wounded and epidermal mucus collected at intervals over the course of wound healing, with extracts of the resulting mucus screened for antimicrobial activity against wound infection pathogens.